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# **STUDIES ON CASPASE SIGNALING IN MICROGLIA**

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# **STUDIES ON CASPASE SIGNALING IN MICROGLIA**

## **THESIS FOR DOCTORAL DEGREE (Ph.D.)**

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*To my family*



# ABSTRACT

The aim of this thesis is to investigate the roles of caspase-8 and caspase-3 in microglia and in brain disorders, beyond their function in apoptosis.

Microglia are resident immune cells of the central nervous system and act as the first line of defense against invading pathogens and other types of brain insults. They are important for normal brain homeostasis and can get rapidly activated upon infection or damage in the brain. However, dysregulated and overactivated microglia have also been reported to cause neurotoxicity and can further contribute to the damage in neurodegenerative diseases.

Caspases are a family of proteases that are important for signaling pathways controlling cell death, inflammation, proliferation and differentiation. Caspase-8 and -3 are well known for their functions in apoptosis, but can also be involved in other processes. We have earlier shown that upon pro-inflammatory stimuli, microglia activate caspase-8 and -3/7 which regulate their activation through a PKC $\delta$ -dependent pathway, without triggering of cell death.

In the first paper we investigate why caspase-3 under some circumstances kills the cells, but under others, like activation of microglia, does not. We show that upon pro-inflammatory stimuli of microglia, cIAP2 regulates the processing of caspase-3 so that it remains in the cytoplasm. Under cell death conditions caspase-3 can be fully processed and thereby enter the nucleus and exert its apoptotic functions.

In order to provide genetic evidence of caspase control of microglia activation, we have created a model in which caspase-8 is conditionally knocked out in microglia. The CASP8 knock-out mice showed a reduction in pro-inflammatory activated microglia, which were associated with reduced neurotoxicity in a mouse model of Parkinson's disease. We are also presenting data on the temporal and spatial activation of caspase-8 and -3 in microglia cells in human subjects who suffered a stroke. In the last paper we screened for genes involved in the metabolism of amyloid precursor protein in patients with late-onset Alzheimer's disease. Significant association was found for rare variants of CASP8 and late-onset of the disease. For two of the variants we performed functional studies, which indicated an altered caspase-8 protein expression and reduced enzymatic activity.

In summary, we have provided genetic evidence for caspase regulation of microglia pro-inflammatory activation and described a mechanism for how caspases prevent killing of cells during activation. We also describe the expression pattern of caspases in microglia cells after stroke, and provide evidence of rare mutations in caspase-8 associated with late-onset Alzheimer's disease.

## POPULÄRVETENSKAPLIG SAMMANFATTNING

Mikroglia är hjärnans egna immunceller. De är viktiga för att försvara hjärnan mot infektioner och hjälpa till att läka den vid skador. Men vid vissa sjukdomar kan de även bli överaktiva och skada nervcellerna, därför är det viktigt att förstå hur de aktiveras och hur de kan regleras.

Kaspaser är en familj av enzymer som reglerar många viktiga processer i cellerna. Kaspas-8 och -3 är vanligtvis aktiva i celldödsprocessen, men vi har i en tidigare studie upptäckt att de även är involverade i aktiveringsprocessen av mikroglia. Men hur kan det komma sig att aktiveringen av samma kaspaser kan leda både till celldöd och till aktivering? Vi har hittat mekanismen för hur det fungerar, vilket är beskrivet in en av artiklarna i denna avhandling.

Vi har även undersökt vad som händer om vi tar bort genen för kaspas-8 enbart i mikroglia i en musmodell. Det ledde till att mikroglia var svårare att aktivera, vilket betyder att kaspas-8 är viktig för aktiveringsprocessen. I en musmodell av Parkinsons sjukdom så såg vi intressant nog att reglerad aktivering av mikroglia även ledde till att färre nervceller dog. I en annan studie har vi visat att kaspas-8 och -3 uttrycks i aktiverade mikroglia-celler efter en stroke. Vi har undersökt vävnad från strokepatienter och kartlagt var och när kaspaserna blir aktiverade. I den sista studien screenade vi en stor grupp patienter med sporadisk Alzheimers sjukdom för att leta efter nya genmutationer. Intressant nog hittade vi två nya ovanliga mutationer i kaspas-8 genen som vi kunde koppla till patienter som utvecklat Alzheimers. Vi kunde även visa att dessa mutationer i kaspas-8 leder till försämrad enzymatisk aktivitet för kaspas-8.

Sammanfattningsvis så har vi här visat hur kaspas-8 och -3 är reglerade och involverade i aktiveringsprocessen av mikroglia samt i hjärnsjukdomar. Denna kunskap är viktig för att bättre kunna förstå hur mikroglia-celler och kaspaser fungerar, samt öka kunskapen om hjärnans sjukdomar.



## LIST OF SCIENTIFIC PAPERS

- I. *Regulation of caspase-3 processing by cIAP2 controls the switch between pro-inflammatory activation and cell death in microglia.*  
Kavanagh E\*, **Rodhe J\***, Burguillos MA, Venero JL, Joseph B.  
Cell Death Dis. 2014 Dec 11;5:e1565.
- II. *Deletion of caspase-8 in mouse myeloid cells blocks microglia pro-inflammatory activation and confers protection in MPTP neurodegeneration model.*  
Kavanagh E, Burguillos MA, Carrillo-Jimenez A, Oliva-Martin MJ, Santiago M, **Rodhe J**, Joseph B, Venero JL.  
Aging (Albany NY). 2015 Sep;7(9):673-89.
- III. *Temporal investigation of caspase-8 activation in myeloid cells in human stroke subjects.*  
**Rodhe J**, Burguillos MA, Kavanagh E, Persson A, Englund E, Deierborg T, Venero JL, Joseph B.  
MANUSCRIPT
- IV. *Association with Alzheimer's disease and functional analysis of rare CASP8 variants.*  
Rehker J\*, **Rodhe J\***, Nesbitt R, Boyle E, Martin B, Lord J, Shendure J, Raskind W, Cruchaga C, Joseph B, Brkanac Z.  
MANUSCRIPT

\*The authors contributed equally to this work

## LIST OF RELATED PAPERS, NOT INCLUDED IN THE THESIS

- I. *p57(KIP2) control of actin cytoskeleton dynamics is responsible for its mitochondrial pro-apoptotic effect.*  
Kavanagh E, Vlachos P, Emourgeon V, **Rodhe J**, Joseph B.  
Cell Death Dis. 2012 May 17;3:e311.
- II. *TAp73 $\beta$ -mediated suppression of cell migration requires p57Kip2 control of actin cytoskeleton dynamics.*  
**Rodhe J**, Kavanagh E, Joseph B.  
Oncotarget. 2013 Feb;4(2):289-97.
- III. *Cell culturing of human and murine microglia cell lines.*  
**Rodhe J**.  
Methods Mol Biol. 2013;1041:11-6.

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## LIST OF ABBREVIATIONS

A $\beta$	Amyloid beta
AD	Alzheimer's disease
ALS	Amyotrophic lateral sclerosis
APC	Antigen-presenting cell
APP	Amyloid precursor protein
Asp	Aspartate
ATP	Adenosine triphosphate
BBB	Blood-brain barrier
BDNF	Brain-derived neurotrophic factor
CARD	Caspase activation and recruitment domain
Caspase	Cysteine aspartic protease
CED-3	Cell death protein 3
CNS	Central nervous system
Cys	Cysteine
CX3CL1	Fractalkine
DAMP	Damage associated molecular patterns
DED	Death effector domain
DISC	Death inducing signaling complex
DR3	Death receptor 3
EGF	Epidermal growth factor
HD	Huntington's disease
His	Histidine
HMGB1	High-mobility group box 1
IAP	Inhibitor of apoptosis
ICE	Interleukin-1 $\beta$ -converting enzyme
IGF-1	Insulin-like growth factor-1
IL	Interleukin
iNOS	Inducible nitric oxide synthase
LPC	Lysophosphatidylcholine
LPS	Lipopolysaccharide

LW	Lewy body
MCP-1	Monocyte chemotactic protein-1
MHCI/II	Major histocompatibility complex I/II
MIP	Macrophage inflammatory protein
MM	Microglia/macrophage
MMP	Matrix metalloprotease
MPTP	1-methyl-4phenyl-1,2,3,6-tetrahydropyrene
MS	Multiple sclerosis
NFkB	Nuclear factor kappa B
NGF	Nerve growth factor
NLR	Nod-like receptor
NO	Nitric oxide
PAMP	Pathogen associated molecular pattern
PARP	Poly (ADP-ribose) polymerase
PD	Parkinson's disease
PDGF	Platelet-derived growth factor
PKC $\delta$	Protein kinase-C delta
pMCAO	Permanent middle cerebral artery occlusion
PRR	Pattern recognition receptor
PSEN	Presenilins
PtdSer	Phosphatidylserine
RAGE	Receptor for advanced glycation end-products
RLR	RIG-I-like receptor
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SN	Substantia nigra
STS	Staurosporine
TGF- $\beta$	Transforming growth factor beta
TIA	Transient ischemic attack
TLR	Toll-like receptor

TNF	Tumor necrosis factor
TRAILR	TNF-related apoptosis inducing ligand receptor
TREM2	Triggering receptor on myeloid cells 2
tPA	Tissue plasminogen activator

# 1 INTRODUCTION

## 1.1 MICROGLIA

### 1.1.1 The brain

The brain is a part of the central nervous system (CNS) and consists of several cell types, which are often roughly divided into neuronal and glial cells. Neuronal cells are arranged in networks (circuits) and communicate with each other via rapid electrochemical signals. The glial cells consist of oligodendrocytes, astrocytes and microglia. The oligodendrocytes form and maintain the myelin sheets that insulate the neuronal axons and improve signal speed. The astrocytes are involved in maintaining the blood-brain barrier (BBB) integrity and the extracellular ion balance, the buffering of neurotransmitters and providing neuronal metabolic support <sup>1</sup>. Microglia are the resident innate immune cells of the brain. They act as the first line of defense against pathogens and contribute to brain homeostasis <sup>2</sup>. The brain is partially separated from the body and the circulating blood flow by the BBB. This diffusion barrier is formed by endothelial cells and pericytes, and hinders influx of most compounds to enter the brain. During inflammation the BBB becomes more permeable, and may allow larger compounds and other immune cells to enter the brain <sup>3</sup>.

### 1.1.2 Microglia

Microglia can be distinguished from the other glial cells by their origin, morphology, functions in the brain and gene expression patterns <sup>2, 4</sup>. Microglia cells are constantly scanning the brain for potential threats, and are rapidly responding to infections or injury. They then undergo a multistage activation process and can acquire different phenotypes. The activated microglia cells have the potential to migrate to the site of infection or injury, undergo proliferation and release pro- or anti-inflammatory compounds and phagocytose cells or debris <sup>2,4</sup>.

Microglia cells were first described by Río-Hortega in 1919 <sup>5</sup>. He gave them their name and classified them as “the macrophages of the brain”. He also described the different morphological appearances the cell can achieve in scavenging, or “resting”, state and upon activation, illustrated by many tissue stainings and drawings. It was also described how the cells were distributed in the brain, and their ability to migrate, proliferate and phagocytose <sup>5,6</sup>.

### 1.1.3 Origin and distribution

The developmental origin of microglia has been a matter of debate for quite some time. Due to the similarities to peripheral monocytes and macrophages, microglia cells were first suggested to be of hematopoietic origin. Although monocytes are able to enter the brain and assume microglia/macrophage morphology, this only occurs under specific conditions when the BBB is impaired. It has now been shown that microglia originate from the embryonic yolk sac during development <sup>7</sup>. They are derived from erythromyeloid progenitor cells that

resemble macrophages and enter the brain via the circulatory system to colonize the brain. Microglia then go through a developmental program where they proliferate and assume the typical microglia ramified morphology and spread throughout the brain <sup>4</sup>.

The number of microglia cells varies depending on the region of the brain. Microglia are reported to represent 5-12 % of all glial cells in the normal mouse brain, with regional density differences <sup>8</sup>. In the normal human brain, microglia represent 0.5 -16.6 % of all brain cells, with substantially higher number of microglia in the white than the grey matter <sup>9</sup>. The population of microglia remains relatively stable through life, and is maintained by self-renewal <sup>10</sup>.

Depending on their location in the brain and the surrounding microenvironment, microglia can differ in morphology with great variation in body size and length and branching of processes even in the normal brain. Microglia are then generally described having a small body and being highly ramified. However, the morphology can alter from more compact and rounded state with short processes at sites lacking the BBB, to microglia cells with long processes following the longitudinal axis of nerve fibers in the white matter, to the radially branched microglia that can be found throughout the grey matter <sup>8</sup>.

#### **1.1.4 Microglia and brain homeostasis**

Although the state of microglia in the healthy brain is sometimes referred to as “resting”, there seems to be no periods of inactivity since microglia processes are highly dynamic and constantly in motion to inspect their microenvironment <sup>11, 12</sup>. The term scavenging or surveying seems to fit the state of microglia in the normal brain better. Besides their role as immune cells, they also contribute to the brain homeostasis. Microglia constantly interact with neurons in the developing brain as well as in the adult brain. If these interactions are disturbed, it can have severe consequences on the brain development and function <sup>4</sup>.

In the postnatal development many new synapse connections are formed, but only some are maintained and others have to be eliminated. Microglia participate in the synaptic remodeling by survey the synaptic maturation and actively engulfing synaptic material <sup>13, 14</sup>. During development and adulthood, microglia also contributes to the formation of neuronal circuits and promote neuronal survival by releasing trophic factors, e.g. insulin-like growthfactor-1 (IGF-1), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), nerve growth factor (NGF), and brain-derived neurotrophic factor (BDNF) <sup>15, 16, 17</sup>.

Microglia also play a role in neuronal cell death. During development, many immature neurons undergo programmed cell death as part of the developmental program. This can be induced by neuronal intrinsic factors as well as factors released from microglia, for example NGF and reactive oxygen species (ROS) <sup>18, 19</sup>. Microglia are also important for the removal of the dead cells throughout life. Phagocytosis of neuronal cells during development as well as removal of dead cells in the adult brain is vital to maintain homeostasis. Without the removal of dead cells and debris, the brain deteriorates and can result in neurological dysfunction <sup>4</sup>. Microglia can participate in the cleanup through phagocytosis, without initiating an



inflammatory response. Non-inflammatory phagocytosis can be mediated via triggering receptor expressed on myeloid cells-2 (TREM2)<sup>4</sup>. Microglia can detect dying cells by their expression of “find me” signals, such as fractalkine (CX3CL1) or lysophosphatidylcholine (LPC), and “eat me” signals, such as phosphatidylserine (PtdSer) or annexin I, which induce the microglial phagocytosis of the cells<sup>20</sup>.

### 1.1.5 Activation

Microglia cells are very plastic and can respond to a large variety of challenges. These cells are equipped with many receptors that can detect a wide range of molecules associated with infection, injury or neurodegeneration. They then undergo a rapid change in gene expression, functional behavior and morphological transformations<sup>2, 4</sup>. This is called microglia activation, although one might argue that this is just a shift in activation state, going from maintenance and surveillance to an immunologically active cell.

The scavenging microglia have a ramified morphology with a small cell body and thin, branched processes. Upon activation, microglia undergo a transformation to different functional states, depending on the cause of activation. Microglia can acquire bushy, rod shaped or various stages of rounded morphologies, with the characteristic macrophage-like amoeboid form with retracted protrusions and enlarged cell body<sup>21, 22, 23</sup>. They become motile and can actively move to the site of injury or infection by following a chemotactic gradient or proliferate at the site upon activation. Microglia activation includes upregulation and rearrangements of surface receptors needed for interactions with surrounding cells and extracellular matrix, changes in intracellular enzymes and release of various inflammatory and immunoregulatory compounds. Microglia can phagocytose damaged cells, tissue debris or microbes, as well as release chemoattractive factors in order to recruit immune cells from the circulation to help fighting infections<sup>2, 4</sup>.

Microglia cells are equipped with innate pattern-recognition receptors (PRRs) that recognize foreign antigens, which allow them to respond to many types of infectious agents, including bacteria, fungi, parasites and viruses. The PRRs include toll-like receptors (TLRs), RIG-I-like receptors (RLRs), NOD-like receptors (NLRs) and C-type lectin receptors which recognize conserved structural motifs of pathogen-associated molecular patterns (PAMPs)<sup>24</sup>. For example, microglia detect bacterial infections through e.g. TLR2, TLR3, TLR4 and TLR9, which recognize the bacterial lipopeptides, polyinosinic-polycytidylic acid, lipopolysaccharides (LPS) and bacterial CpG DNA respectively<sup>25</sup>. Upon activation, microglia can become phagocytes and secrete many proinflammatory cytokines, including TNF, IL-1 $\beta$ , IL-6 and inducible nitric oxide synthase (iNOS), reactive nitrogen (RNS) and oxygen species (ROS) to kill the bacteria<sup>26, 27, 28</sup>. They also release chemoattractants, such as monocyte chemoattractant protein1 (MCP-1) and macrophage inflammatory protein-2 (MIP-2) to recruit immune cells from the periphery to clear the infection<sup>29, 30</sup>. Viral agents are also recognized by the PRRs. They induce an acute inflammatory response with release of many proinflammatory cytokines (IFN- $\beta$ , IFN- $\gamma$ , TNF, IL-1 $\beta$ , IL-6, IL-12), chemoattractants as well as activation of microglial antigen-presenting functions. Microglia are able to upregulate

co-stimulatory molecules like CD40 and CD86, and major histocompatibility complex I and II (MHC-I/II). They also have the ability for antigen presentation to infiltrating adaptive immune cells, but do not seem to be as efficient as professional antigen presenting cells (APCs)<sup>4, 31, 32</sup>.

In addition to the PRRs, microglia also express many other types of receptors that contribute to the recognition of damage associated molecular patterns (DAMPs) released in high amounts from damaged or dying cells. For example, detection of the DAMP signal adenosine triphosphate (ATP) by purinergic receptors or high-mobility group box 1 (HMGB1) by TLR2, TLR4 and receptor for advanced glycation end-products (RAGE) rapidly activates microglia<sup>33, 34</sup>. In conditions like traumatic brain injury or ischemic damage, large amounts of DAMPs are released from dying cells and attract microglia to the site of the injury<sup>12</sup>. They trigger an acute inflammatory reaction with release of inflammatory mediators and phagocytosis. However, if the inflammation is sustained and reaches a chronic phase, this can lead to deleterious effects on the neuronal population. If the inflammation is not resolved after clearance of the damaged area, the chronically activated microglia continue to release cytotoxic compounds and induce oxidative stress. This cause more neuronal loss, and have been detected in several neurodegenerative diseases<sup>35, 36</sup>. Microglia have also been shown to induce neuronal death by phagoptosis. Under inflammatory conditions, microglial release of soluble mediators, such as peroxynitrite or TNF, can induce neuronal phosphatidylserine exposure and lead to microglial phagocytosis of the stressed-but-viable neurons<sup>37, 38</sup>.

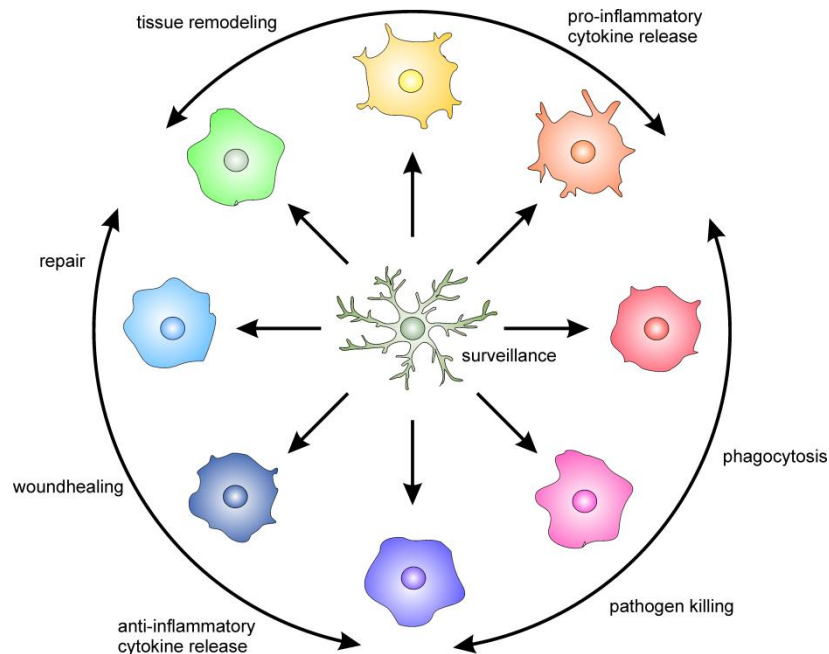
### 1.1.6 Phenotype classification

The generalized classification system for describing macrophages is also commonly used for microglia. Although simplified, it can be a useful tool to describe the type of activation the cells are undergoing. Under physiological conditions, microglia are in scavenging state, surveying the local microenvironment. Disturbance in brain homeostasis can induce microglia activation, for which the activation stages are typically described as classical activation (M1) and alternative activation (M2)<sup>22</sup>.

The classical activation is a defensive state and a typical response to an infectious agent. Microglial activation by e.g. IFN $\gamma$ , LPS or other TLR activators are common, and results in release of high amounts of pro-inflammatory cytokines (e.g. TNF, IL-1 $\beta$ , IL-18) and oxidative metabolites (ROS, NO), enhanced microbicidal capacity and expression of co-stimulatory molecules<sup>22, 39, 40</sup>.

The alternative activation is more of a reparative, matrix remodeling and anti-inflammatory response. It is characterized by release of anti-inflammatory cytokines (e.g. IL-10) and matrix metalloproteases (e.g. MMP1 and -12) upon e.g. IL-4 or IL-13 recognition. This category has been further divided into functions, related to tissue repair and wound healing, as well as a state of acquired deactivation<sup>22, 39, 40</sup>.

Lately it has been discussed that it is more likely that there is a whole spectrum of activation states, and the cell can shift between activation states either based on the type of stimulus or the previous activation state (Figure 1) <sup>40, 41</sup>.



**Figure 1.** Microglia have a broad spectrum of activation states that they can shift between.

### 1.1.7 Microglia markers

As for today, there is not one single marker that is ideal for detecting microglia in tissue. In the intact CNS, it is possible to distinguish microglia from the surrounding cells by e.g. the widely used Ionized calcium-binding adapter molecule-1 (Iba1) that mark all microglia, with an increased expression upon activation <sup>42, 43</sup>. Other general markers used for microglia are Cd11b/MAC1/OX42, F4/80, IB4 and tomatolectin. A frequently used marker for activated microglia with lysosomal activity is CD68. There are also several markers that can be used for subsets of activated microglia, like MHCII, complement receptors or scavenger receptors <sup>2, 22, 39</sup>. In pathologic conditions with breakdown of the BBB, blood derived macrophages can enter the brain. They express very similar spectrum of receptors as microglia, and it is problematic to distinguish between the two cell types. In experimental studies, double-transgenic mice with CX3CR1-GFP labeled microglia and CCR2-RFP labeled macrophages can be used. Here it is possible to distinguish the two cell types, even under pathological conditions when macrophages have entered the brain and work together with microglia <sup>44, 45</sup>.

## **1.2 NEURODEGENERATIVE DISORDERS**

### **1.2.1 Microglia in disease**

Microglia activation is important for host defense and to resolve diseases of the brain. However, dysregulated and overactivated microglia can induce detrimental neurotoxic effects by production of cytotoxic factors which contribute to neuronal cell death <sup>36</sup>. In several neurodegenerative disorders, high numbers of microglia are detected. For example in Alzheimer's disease (AD) and Parkinson's disease (PD), protein aggregates and dying neurons can activate microglia and result in chronic inflammation and neuronal loss <sup>46</sup>. Microglia have also been linked to pathology and disease progression in other neurodegenerative disorders such as multiple sclerosis (MS), amyotrophic lateral sclerosis (ALS) and Huntington's disease (HD) <sup>36</sup>. After events like stroke or traumatic brain injury, an acute inflammation is initiated with microglia and additional cells from the periphery. If the inflammation is not resolved, microglia can enter a chronic activation stage releasing high amounts of inflammatory mediators, resulting in neuronal death <sup>47</sup>. When it comes to brain tumors, another problem arises when microglia switch to a tumor supporting phenotype. Instead of eliminating the tumor cells, microglia have been shown to promote tumor growth and invasion of the surrounding tissue <sup>46</sup>. The following sections focus on brain disorders that are covered in the thesis.

### **1.2.2 Stroke**

Stroke is one of the leading causes of death today, and many of the survivors suffer from permanent disabilities. The incidence of stroke rises exponentially with age. Because of the aging populations in the western world, stroke-related disability and increased need of healthcare is a growing problem <sup>48</sup>. There are several common risk factors for stroke that are associated with health and life style, e.g. hypertension, diabetes and smoking. Risk factors such as atrial fibrillation and transient ischemic attacks (TIA) are less prevalent, but also more specific than the general health and life style factors <sup>48</sup>.

Stroke is caused by a transient or permanent disturbance in cerebral blood flow, which limits the blood supply to a part of the brain. Stroke can be caused by two different mechanisms, which are classified as either hemorrhagic or ischemic stroke <sup>48, 49</sup>. 15% of all stroke cases in North America and Europe are considered to be due to intracerebral hemorrhage. It is commonly caused by hypertensive small-vessel disease, when small aneurysms ruptures and causes a bleeding in the brain. It can also be due to intracranial vascular malformations, cerebral amyloid antipathy or secondary hemorrhage in a previous infarct <sup>50</sup>. Ischemic stroke is the most common type of stroke, and about 85% of all incidences fall into this category. Ischemic stroke is caused by an occlusion of a blood vessel, resulting from thrombosis or embolism (e.g. blood clot blockage formed at the site or distal) or global ischemia (e.g. by cardiac arrest) <sup>49, 51</sup>.

The sudden drop in blood flow leads to acute oxygen- and glucose deprivation in the affected area, called the ischemic core. The most instant and severe damage of the tissue takes place

here in this area, where necrosis can be detected already within a few minutes<sup>48, 49</sup>. The surrounding area is called the ischemic penumbra. It has reduced perfusion and some functional impairment, but has still structural integrity and can survive longer than the core. Within the ischemic area a series of events begins with energy depletion, disturbance in ion homeostasis, glutamate release, calcium channel dysfunction, release of free radicals, membrane disruption and other changes which triggers necrosis and apoptotic cell death<sup>48, 49</sup>. The danger signals released from injured and dying cells triggers the immune system, which is rapidly activated and plays an important role in the post-ischemic damage of tissue and secondary neurodegeneration<sup>48, 52</sup>.

Within the first minutes-hours after ischemic stroke (the acute phase), resident microglia cells are activated and release pro-inflammatory cytokines, e.g. tumor necrosis factor (TNF) and interleukin-1 $\beta$  (IL-1 $\beta$ ), nitric oxide (NO) and chemokines, e.g. monocyte chemoattractant protein-1 (MCP-1/CCL2) and macrophage inhibitory factor 1 $\alpha$  (MIP-1 $\alpha$ /CCL3)<sup>47, 53</sup>. They are soon accompanied by infiltrating inflammatory cells from the circulation. In both animal models and in tissue from human stroke patients, immune cells such as neutrophils, T-lymphocytes and monocytes/macrophages are present and contribute to the inflammation around the ischemic area<sup>52, 54, 55</sup>. In this sub-acute phase (1-5 days after stroke), the infarct is spread to the penumbra. Cellular damage is triggered by excitotoxicity, mitochondrial disturbances, as well as the production of reactive oxygen species and pro-inflammatory cytokines from the immune cells, which are contributing to the tissue injury and secondary neurodegeneration<sup>47, 55</sup>. In the late/chronic phase (weeks after stroke), the number of immune cells decrease. Beside the pro-inflammatory activity of microglia, they also contribute to tissue recovery by scavenging necrotic debris and limiting inflammation by secretion of anti-inflammatory cytokines, e.g. interleukin-10 (IL-10) and TGF- $\beta$ <sup>55, 56, 57</sup>.

Current treatment of patients with acute stroke is intravenous tissue plasminogen activator (tPA) if the patient arrives to the clinic within 3-4.5 hours after stroke onset or aspirin if within 48 hours. The phrase “time is brain” is often used to stress the importance of receiving immediate treatment to save the cells at risk in the ischemic penumbra before irreversible injury occur. Depending on the cause of the stroke, additional treatments are used, such as decompression surgery, antiplatelet agents, endarterectomy or mechanical thrombectomy<sup>48, 58</sup>.

### 1.2.3 Alzheimer's disease

Alzheimer's disease (AD) is a progressive neurodegenerative disorder and the most common form of dementia worldwide. Elderly people are mostly affected (late onset AD), but 4-5% of all cases develop the disease before the age of 65 (early onset AD). The aging population is also accompanied with increased prevalence of dementia worldwide, a problem that seems likely to grow even more in the coming years<sup>59, 60</sup>. AD is clinically manifested by mild and gradual memory deficits, which irreversibly advance over time, and decline in cognitive function. With the progress of the disease follows manifestations in language disorders, visuospatial deficits and executive dysfunctions<sup>61</sup>. The exact cause of the disease is not well

understood, however the most prevalent pathological hallmarks of AD is the extracellular accumulation of amyloid-beta ( $A\beta$ ) aggregates (amyloid plaques) and intracellular aggregates of neurofibrillary tangles (tau) together with extensive loss of cortical neurons and synapses<sup>62</sup>. The “amyloid hypothesis” describes  $A\beta$  as key factor in triggering the AD neurodegenerative cascade, with cerebral accumulations of the amyloid precursor protein (APP) cleavage products, the  $A\beta$ 1-41 and  $A\beta$ 42 peptides. Formations of  $A\beta$  oligomers and aggregation into neurofibrillary tangles together with hyperphosphorylated tau disrupts the structure and function of the neurons and leads to progressive cognitive decline. Additionally, the aggregates trigger a chronic inflammatory response, which also plays a role in the disease progression<sup>63, 64</sup>. Several neuroinflammatory mediators are released by both microglia, astrocytes and neuronal cells in AD brains<sup>65</sup>.

Imaging studies have shown microglia localized around  $A\beta$  plaque in the brain of patients and in animal models of AD<sup>66, 67</sup>. Microglia are involved in the  $A\beta$  clearance by phagocytosis and secretion of enzymes involved in  $A\beta$  degradation. However, in the late stages of AD, the clearance seems very slow and inefficient<sup>68, 69</sup>. Even so, microglia activation seems to increase throughout the disease progression<sup>70</sup>. The  $A\beta$  plaques are not just toxic to the neurons; together with the signals from the dysfunctional or dying neurons they are also activating microglia. Uncontrolled and chronic activation of microglia cells may also contribute to neuronal injury. The activated microglia are releasing inflammatory mediators, including cytokines, chemokines, NO, free radicals, complement factors and prostaglandins which could eventually contribute to additional neural toxicity and cell death<sup>36, 69, 71</sup>.

There is a strong genetic component in AD, with some gene mutations and polymorphisms correlated to the disease. There are several known mutations in genes associated with APP or APP processing. The most prevalent mutations are in the APP gene itself, or presenilins PSEN1 and PSEN2, which are associated with familial early onset AD<sup>63, 72</sup>. In addition, triggering receptor on myeloid cells 2 (TREM2) that is expressed on microglia has also been associated with early-onset AD. Most of the AD cases are however sporadic, and several potential risk genes are known. The most consistently associated gene is APOE, but several phosphokinases like GSK3 $\beta$  and DYRK1A, and the gene encoding Tau are also identified as risk genes<sup>63, 73</sup>. Other risk factors for developing AD are age and head injuries, but also modifiable life style factors like food intake, physical activity, cognitive reserve, alcohol intake and smoking have been found<sup>63</sup>.

AD can be difficult to distinguish from other dementias at the clinic, and no definite diagnosis can be performed today. The only way to confirm the diagnosis is post mortem tissue analysis. Available treatments for AD are mainly focusing on the symptoms, and include cognitive training, symptomatic treatment for cognitive decline, as well as usage of antidepressants and antipsychotic drugs when needed<sup>63</sup>.

#### 1.2.4 Parkinson's disease

Parkinson's disease (PD) is the second most common neurodegenerative disorder, with a prevalence about 1% at the age of 60, and up to 4% at age 85 <sup>74</sup>. PD is a chronic and slowly progressive disorder characterized by clinical manifestations of resting tremor, bradykinesia (slowed movements), rigidity and postural instability. The motor symptoms are linked to the loss of dopaminergic neurons in substantia nigra (SN) and the subsequent dysfunction of the basal ganglia, which are involved in initiation and execution of movements. As the disease progresses, other problems like dyskinesia (involuntary movements), pain, impaired olfaction, sleep problems, autonomic dysfunctions and neuropsychiatric manifestations (dementia, depression and hallucinations) appear <sup>75</sup>.

Although most cases of PD are idiopathic, some familial forms of hereditary PD exist with several identified susceptibility genes e.g. SNCA ( $\alpha$ -synuclein) and PARK2 (parkin) <sup>75</sup>. Risk factors for PD are advanced age, male gender, European ancestry and exposure to environmental toxins. Habits like smoking and coffee consumption on the other hand is associated with reduced risk for PD <sup>74</sup>.

The degeneration of the dopaminergic neurons in SN is consistent in a neuropathologic examination, however different protein pathologies can be detected, like  $\alpha$ -synucleinopathy or tauopathy. Development of spherical Lewy bodies (LB), which are  $\alpha$ -synuclein aggregation into insoluble fibrils, and  $\alpha$ -synuclein accumulations within neurites (Lewy neurites) are common features in PD <sup>76</sup>.

Astrogliosis, microgliosis and increased levels of pro-inflammatory mediators in the blood and cerebrospinal fluid are often detected in PD patients and PD animal models <sup>77</sup>. Accumulation of  $\alpha$ -synuclein is toxic to dopaminergic neurons and also cause activation of microglia cells through PRRs. Additionally, dying and dead neurons also trigger microglia activation, leading to sustained activation of microglia and release of inflammatory mediators, including TNF, IL-1 $\beta$ , IL-6 and ROS. Chronic activation of microglia and release of toxic factors can lead to additional loss of neurons, thereby causing a viscous cycle of neuronal degeneration <sup>78</sup>. Cases with patients who administered the toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), or animal models based on MPTP or LPS injections, shows an acute inflammatory response with microglial activation and release of toxic inflammogens followed by the death of dopaminergic neurons. The animal models showed that the inflammation preceded cell death, and thereby causative of the neuronal loss <sup>79, 77</sup>.

Diagnosis of PD cases is mainly based on symptoms, with additional tests for confirmation, such as neuroimaging. When the motor symptoms are clinically recognized, about 50-60% of the dopaminergic cells in SN are already lost, resulting in 80% depletion in striatal dopamine <sup>80, 75</sup>. The most common treatment for PD is levodopa as replacement therapy to treat the motor symptoms. Other treatments can be used, such as dopamine agonists, MAO-B inhibitors, COMT inhibitors, surgical intervention and deep-brain stimulation <sup>81</sup>.

## 1.3 CASPASES

### 1.3.1 Caspases

Caspases, **cysteine-aspartic proteases** or **cysteine-dependent aspartate-specific proteases**, are a family of proteases (family C14; clan CD) that are part of the signaling pathways controlling cell death, inflammation, proliferation and differentiation. As their name implies, they carry out their enzymatic properties by recognizing substrates containing aspartate (Asp), where they use the cysteine (Cys) residue in their active site to catalyze a peptide bond cleavage in the target protein. Even though many proteases use Cys to catalyze peptide-bond hydrolysis, the caspase specificity for Asp containing substrates is quite rare for proteases<sup>82, 83</sup>. Caspases are expressed in the cells as inactive precursors which are tightly regulated. That means they can get rapidly activated upon a specific stimulus to perform a limited proteolysis, which in turn activates cascades of events leading to different biological responses, depending on the initial stimulus<sup>82</sup>.

Caspases are evolutionary conserved from the nematode worms to mammals. The first caspase was found in mammalian cells in 1992 as the interleukin-1 $\beta$ -converting enzyme (ICE), the inflammatory protease we today call caspase-1<sup>84, 85</sup>. The year after, cloning of the cell death gene CED-3 in *Caenorhabditis elegans* and was shown to have important functions in programmed cell death. Since CED-3/ICE-like proteases were structurally similar to caspase-1, they proposed that cysteine proteases also could have apoptotic functions in mammals<sup>86</sup>. Soon the field exploded, as more caspases were identified. Gene deletion studies and biochemical *in vitro* experiments were done to characterize them, which especially contributed to new developments in the field of cell death. New mechanisms and functions for the individual caspases emerged, and they still do<sup>87</sup>.

### 1.3.2 Structure and mechanism of activation

Caspases are synthesized as inactive single chain polypeptide precursors (zymogens). They contain an N-terminal pro-domain followed by a large and a small catalytic subunit, which can be divided by a linker peptide (Figure 2). The large peptide is generally composed by ~20 amino acids, while the smaller subunit is ~10 amino acids<sup>88</sup>.



**Figure 2.** Overview of general caspase structure, including pro-domain, large- and small catalytic subunit.

The N-terminal domain in the caspases is important for protein binding, and can contain a caspase recruitment domain (CARD) or death effector domain (DED). They mediate dimerization or binding to larger complexes in the first step of activation. The large subunit contains a catalytic dyad of Cys and histidine (His), while the small subunit contains several residues that form the substrate binding groove<sup>82</sup>.

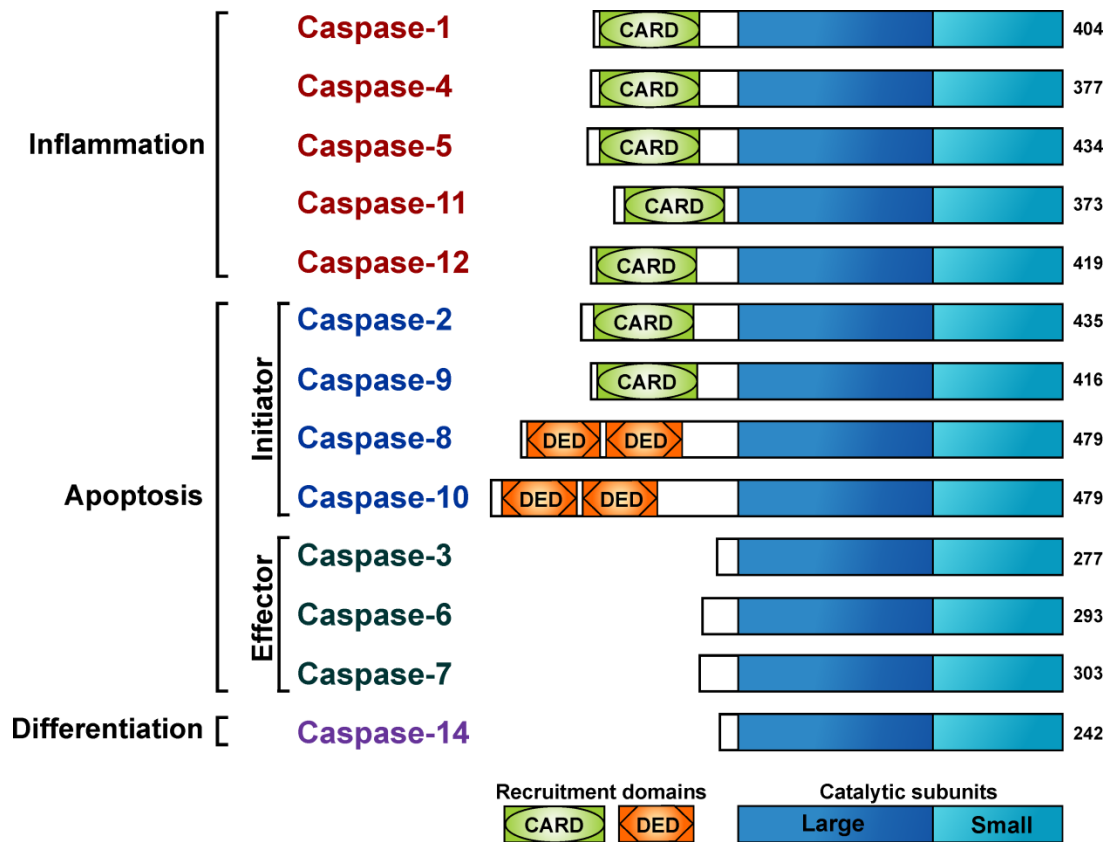


The apoptosis related caspases are usually subdivided into initiators and executioners. These groups are activated by different mechanisms. The activation of initiator caspases is triggered by homodimerization of the caspase monomers and mediated by scaffold proteins. Upon apoptotic stimuli, caspases are recruited to activation platforms where adaptor proteins can bind to the CARD or DED domains. Homodimerization of caspases leads to auto-proteolytic cleavage of the bond between the small and the large subunits, and a subsequent cleavage between the pro-domain and the large subunit<sup>82, 89</sup>.

The effector caspases have shorter pro-domains and exists as inactive pro-caspase dimers. They can get activated by cleavage by the initiator caspases, which cleave the bond between the large and the small subunits. This allows a conformational change of the complex, bringing the two active sites of the dimer together to a mature functional protease. The activated executioners can then cleave and activate other executioner caspases, resulting in an accelerated activation cascade, and their other physiological substrates<sup>82, 89</sup>.

### 1.3.3 Nomenclature and specificity

To date, up to 14 mammalian caspases have been identified, whereof 12 of them exist in human (Figure 3). Caspases are widely spread in the different cell types and tissues, with the exception of caspase-14 that is only expressed in keratinocytes<sup>90</sup>. The human caspases includes caspase 1-10, -12 and -14<sup>87, 91</sup>.



**Figure 3.** Schematic representation of caspase 1-14, grouped according to classification. Number of amino acids is indicated to the right. CARD and DED recruitment domains are illustrated in green and orange. Large and small catalytic subunits are illustrated in blue.

Some caspases are not present in all mammals, for example caspase-11 is a murine enzyme that is likely a homologue of the human caspase-4 and -5. The protein that was originally named caspase-13 was later found only to be a bovine homologue of caspase-4. Moreover, the human caspase-5 is not expressed in mice. Caspase-12 exists both as a full-length and a truncated alleles in humans, but only in the full-length form in rodents<sup>92, 93, 94</sup>. In evolution, there is a tendency towards increased number of caspases over phylogenetic time, ranging from four in *C. Elegans* to seven in *Drosophila* and 11/12 in mice and human<sup>95</sup>.

Caspase substrate specificity is mainly determined by 4 continuous residues on the target protein (P4-P3-P2-P1). The P1 position consists of Asp, after which the protein is cleaved on the C-terminal side of the sequence<sup>96</sup>. The preferred residue at P3 is Glu for all the mammalian caspases, but other residues can also be used in this position. The P4-P2 residues will interact with the caspase catalytic groove, and therefore the specificity for the P4-P2 positions varies between different caspases. For example caspase-2, -3, -7 are efficient in cleaving peptides with the DEVD peptide sequence, but much worse in cleaving a WEHD substrate. Conversely, caspase-1, -5 have the opposite affinity to the same target sequences. Moreover, caspase-6, -8, -10 prefer (V/I/L)ETD sequences, but caspase-9 prefer LEHD. One caspase can however have several more or less efficient target sequences, and several caspases can overlap in target specificity<sup>82, 97</sup>.

#### **1.3.4 Caspase classification**

Classification of caspases is traditionally done according to function, to pro-peptide length (related to activation mechanism) or based on synthetic substance preference. The functional classification of mammalian caspases is often used, with division into three major functional categories; pro-apoptotic caspases, pro-inflammatory caspases and caspases involved in keratinocyte differentiation (Fig3). However, the classification has become more complicated since new functions for many caspases are constantly discovered, including proliferation, differentiation, cell motility and tissue invasion. Also, the same caspase can have functions of both apoptotic and anti-apoptotic character<sup>98</sup>.

The pro-apoptotic caspases have been discovered for their active role in cell death pathways. They are further classified into initiators (including caspase-2, -8, -9, -10) and executioners (caspase-3, -6, -7). Initiator caspases activate executioner caspases, which in turn can cleave many cellular proteins to trigger apoptosis. However, many non-apoptotic functions have been assigned to these caspases as well. The pro-inflammatory caspases are involved in maturation of cytokines in innate immune cells. In addition, an inflammatory response to infection by programmed cell death (pyroptosis) also exist, which includes caspase-1 processing of cytokines. Caspase-14 has not been shown to play a significant role in cell death or inflammation and form the third class. It is expressed in epidermis and is involved in keratinocyte differentiation<sup>82, 88</sup>.

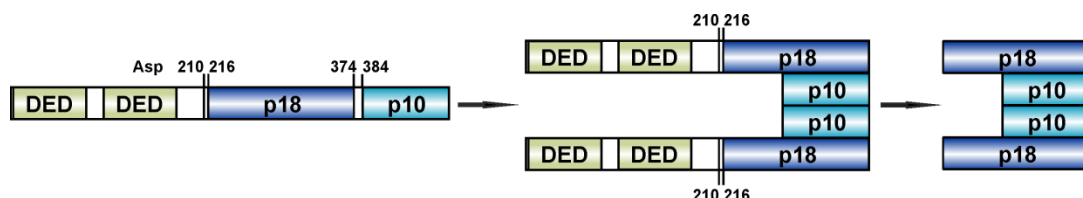
### 1.3.5 Regulation of caspases

Caspases exist as zymogens in the cells, and their processing and activity needs to be tightly regulated. Uncontrolled activation of caspases could have deleterious effects, resulting in death of the cell. Several proteins are associated with caspase regulation, and can act by direct inhibition or bind to adaptor proteins to hinder the caspase-adaptor protein interaction. The first identified endogenous caspase inhibitors were the human inhibitors of apoptosis (IAPs). The X-linked IAP (XIAP), and the cellular IAPs, cIAP1 and cIAP2, consist of three baculoviral IAP repeats (BIR) domains that mediate protein-protein interactions and a RING domain that confers E3 ubiquitin ligase activity. XIAP is a potent inhibitor of caspase activity, and acts by direct binding to the caspase. The BIR2 domain inhibits caspase-3 and -7, whereas BIR3 inhibits caspase-9 activity<sup>99, 100</sup>. cIAP1 and cIAP2 also contain a CARD domain, but the mechanism for regulation of caspase activity is not fully understood. The cIAPs can bind to the effector caspases, but do not directly inhibit the proteolytic activity. IAPs also have other cellular target proteins, and can regulate both apoptotic and inflammatory pathways. Other members of the IAP family include NAIP, ML-IAP, ILP2, Bruce and Survivin<sup>100, 101</sup>.

Caspase activation can also be regulated by e.g. the viral inhibitor CrmA and p35/49, or the decoy proteins FLIP, COP-1, INCA and ICEBERG, that hinders recruitment to activation platforms, or by post translational modifications<sup>82</sup>.

### 1.3.6 Caspase-8

Caspase-8 can be expressed as 9 different isoforms in human, whereof isoform 1 and 4 are the predominant expressed forms in the cells. Isoform 1 consists of 479 amino acids and is 55 kDa and isoform 4 of 496 amino acids and is 58 kDa<sup>102</sup>. The procaspase-8 consists of a long N-terminal domain, containing two DED domains, followed by a large and a small catalytic subunit (Figure 4). The zymogen is proteolytically processed at Asp<sup>210</sup>, Asp<sup>216</sup>, Asp<sup>374</sup> and Asp<sup>384</sup> (isoform 1) to generate a tetramer consisting of two small and two large subunits. The first cleavage generates the p43/41 intermediate fragments, followed by additional cleavages into p26/24, p18 and p10 fragments<sup>103</sup>.



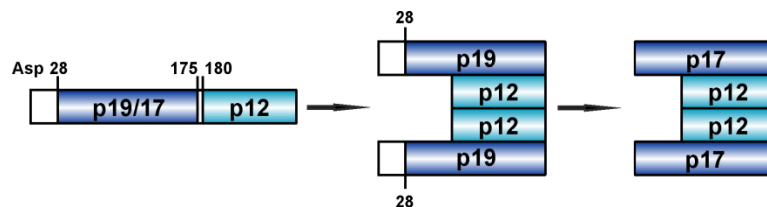
**Figure 4.** Caspase-8 structure and processing, with indicated Asp cleavage sites.

Caspase-8 is predominantly known for its function as an apical caspase in apoptotic pathways. The extrinsic pathway of apoptosis is triggered by extracellular activation of death receptors. They are members of the TNF superfamily, and includes TNFR1, FasR (CD95) death receptor 3 (DR3) and TNF-related apoptosis inducing ligand receptor-1/2 (TRAILR). Binding of respective ligand to the receptor induce receptor oligomerization and recruits

procaspase-8 via the DED to the death inducing signaling complex (DISC) formed at the intracellular part of the receptor. The DISC complex also includes adaptor proteins, such as FADD, TRADD, TRAF or RIP. Dimerization and activation of caspase-8 can initiate apoptosis by direct cleavage of executioner caspases or through the intrinsic apoptotic pathways by e.g. cleavage of Bid. Bid and other proteins facilitate cytochrome c release from the mitochondria into the cytoplasm. Cytochrome c interacts with APAF1, ATP and caspase-9 to form the apoptosome, resulting in caspase-9 activation and subsequent activation of executioner caspases<sup>104</sup>. Depending on the strength and duration of the stimuli, the type of death receptor and adaptor proteins, the activation can also initiate other cellular processes. Apart from the role in apoptosis, caspase-8 can also promote cell differentiation, regulate cell proliferation, regulate autophagy, promote cellular migration and immune signaling<sup>105, 106, 107</sup>. Caspase-8 has also been shown to regulate necroptosis (a regulated form of necrosis) by suppressing RIP1/RIP3. Inhibition of caspase-8 in inflammatory activated microglia can induce necroptosis, however not in neurons, astrocytes or unactivated microglia<sup>108, 109</sup>.

### 1.3.7 Caspase-3

Human caspase-3 is expressed as a 277 amino acids long propeptide of 32 kDa<sup>110</sup>. It consists of a short N-terminal domain, followed by a large and a small catalytic subunit, which are processed at Asp<sup>28</sup>, Asp<sup>175</sup> and Asp<sup>180</sup> (Figure 5). The zymogen is first cleaved into two heterodimer complexes consisting of the p19/p12 subunits, followed by autocatalytic cleavage of the p19 resulting in the p17/12 tetramer<sup>111</sup>.



**Figure 5.** Caspase-3 structure and processing, with indicated Asp cleavage sites.

The traditional view of caspase-3 is as executioner of apoptosis. For example caspase-8 mediated cleavage of caspase-3 in the extrinsic apoptotic pathway, or caspase-9 mediated cleavage in intrinsic apoptotic pathway results in activation of caspase-3. Activated caspase-3 can then cleave specific cellular substrates which results in demise of the cell<sup>104</sup>. Poly (ADP-ribose) polymerase (PARP) is a well-known substrate for caspase-3. Cleavage of PARP facilitates cellular disassembly and can be used as a marker to detect apoptosis<sup>112</sup>.

Caspase-3 is also involved in several non-apoptotic processes. For example, low levels of active caspase-3 are needed for some of the synaptic changes that underlie memory in neuronal cells<sup>113</sup>. Caspase-3 is involved in neuronal differentiation, migration and maintenance of stem cell quiescence<sup>114</sup>. Amyloid precursor protein can be cleaved by caspase-3, which generates a small fragment called C31. Caspase-3 is also important for the differentiation of many other cell types and is involved in inflammatory signaling<sup>107</sup>.

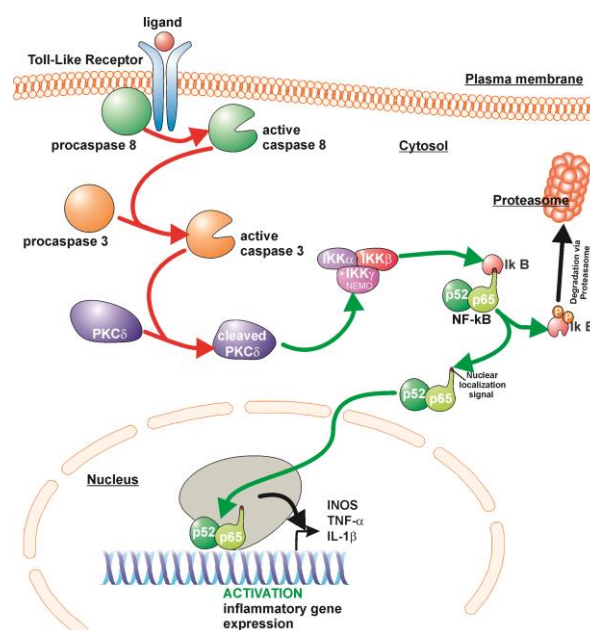
### 1.3.8 Caspase signaling in microglia and inflammation

Caspase-1 is well known for its role in inflammation. Activation of NLRs leads to inflammasome formation, and includes maturation of caspase-1. The activated caspase-1 mediates processing and maturation of IL-1 $\beta$  and IL-18, which are released during inflammation<sup>115</sup>.

Apart from their traditional roles in apoptosis, caspase-3 and -8 have been associated with inflammatory signaling. Lately, caspase-8 has been shown to be involved in inflammasome modulation and also in direct IL-1 $\beta$  regulation<sup>115</sup>. Moreover, caspase-8 is required for T and B cell clonal expansion and for lymphocyte homeostasis and immune function. Caspase-3 has also been linked to lymphocyte activation and B cell differentiation<sup>116, 107</sup>.

Interestingly, caspases have been associated with macrophage differentiation. Caspase-3, -8 and -9 were shown to contribute to the differentiation of monocytes to macrophages. Caspase-8 has also been linked to prevention of sustained activation of nuclear factor kappa B (NF $\kappa$ B) during differentiation and maintenance of macrophage activation<sup>117, 118</sup>.

Several studies in this thesis have been inspired by our earlier finding of caspases controlling the activation of microglia cells<sup>119</sup>. Microglia stimulated with various inflammogens showed activation of caspase-8 and caspase-3/7, in the absence of cell death both *in vivo* and *in vitro*. The orderly activation of caspase-8 and caspase-3/7 lead to activation of NF $\kappa$ B pathways through the processing and activation of protein kinase-C $\delta$  (PKC $\delta$ ), and result in transcription of pro-inflammatory cytokines (Figure 6). Inhibition or knockdown of the caspases hindered microglia activation and release of the inflammatory mediators. Moreover, these caspases could also be put in the context of neurodegenerative disorders, where detection of caspase-8 and -3 were found in activated microglia in patients with AD and PD<sup>119</sup>.



**Figure 6.** Pro-inflammatory activation of microglia leads to caspase-8 and -3/7 cleavage and activation of NF $\kappa$ B pathways.

## 2 AIMS OF THE THESIS

The overall aim of the thesis was to investigate the roles of active caspase-8 and caspase-3 in microglia (mainly), beyond their function in apoptosis.

**Paper I:** In the first paper we were interested in understanding how activation of caspase-3 in some situations can lead to activation of microglia cells, while in other circumstances can lead to cell death.

**Paper II:** Using a conditional knock-out of the CASP8 gene in microglia cells, we wanted to investigate the effect on microglia activation and the possible beneficial effect on neuronal survival in mouse models of Parkinson's disease.

**Paper III:** The expression of cleaved caspase-8 and -3 in activated microglia cells were investigated in post mortem tissue from stroke patients and in a mouse model of stroke to find the temporal and spatial activation patterns.

**Paper IV:** In a screening for proteases related to processing of amyloid precursor protein we found mutations in CASP8 in patients with late onset Alzheimer's disease. We are here investigating if the mutated forms of caspase-8 have functional differences from the wildtype caspase-8 *in vitro*.

## 3 RESULTS

### 3.1 PAPER I.

#### **Regulation of caspase-3 processing by cIAP2 controls the switch between pro-inflammatory activation and cell death in microglia.**

A previous finding of the group showed a novel role for caspases in the microglia activation process. Caspase-8 and -3 activation were shown to regulate microglia pro-inflammatory activation, in the absence of cell death<sup>119</sup>. But this discovery also raised many questions; how can these caspases, which are commonly known for their role in apoptosis, become activated without killing the cells? What is preventing them from their apoptotic functions?

To answer these questions, we used the BV2 microglia cell line to compare the activation and cell death pathways. Upon treatment with lipopolysaccharide (LPS) to induce pro-inflammatory activation or staurosporine (STS) to induce cell death, we found a difference in the cleavage pattern of caspase-3. Caspase-3 is processed in two steps; the first cleavage generates the p19/p12 complexes, which are thereafter further processed into p17/p12 complexes. After activating the cells we could only detect the first step of caspase-3 processing into the p19 form (p19/p12 complex), but with the cell death stimuli we could also detect the fully processed form, p17 (p17/p12 complex). Interestingly, p17 was also detected in the nuclei upon cell death stimuli, while p19 was retained in the cytoplasm in both conditions.

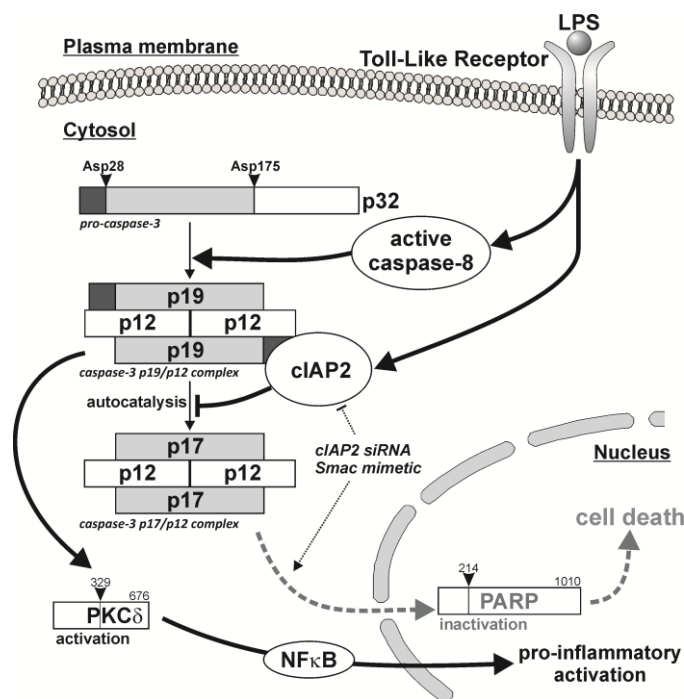
But what is hindering the processing from the p19 to p17 form in the activated microglia? We started by investigating if any of the caspase inhibitors of the IAP family were involved. After microglia activation we found an increase in cIAP2 both on mRNA and protein levels in the cells, but not of XIAP or cIAP1. Could it be that cIAP2 is hindering the full processing of caspase-3 in activated cells? Indeed, using proximity ligation assay we could detect an increase in interactions between cIAP2 and cleaved caspase-3 upon microglia activation. Using subcellular localization assay we could also confirm the presence of cIAP2 in the cytoplasm, where we have previously detected the p19 form of caspase-3.

To confirm that cIAP2 is hindering the p19 to p17 conversion and retain p19 in the cytoplasm, we knocked down cIAP2. Indeed, knockdown of cIAP2 in the activated cells lead to an increased p19 to p17 conversion and caspase-3 activity. The localization of active caspase-3 can regulate the ability to process cytoplasmic substrates, like PKC $\delta$  during microglia activation, or nuclear, like PARP under cell death conditions. After cIAP2 knockdown or STS treatment we did indeed find PARP cleavage, indicating that cIAP2 is hindering the caspase-3 to induce cell death during microglia activation. Interestingly, LPS treatment after knockdown of cIAP2 did not just induce cell death, but also hindered microglia activation.

Finally, using the compound BV6 to reduce the cIAP2 and caspase-3 interaction we confirmed the results obtained with cIAP2 knock down. BV6 was able to suppress the

inhibitory effect cIAP2 has on the full processing of caspase-3, leading to reduced microglia activation and increased cell death.

In summary, cIAP2 regulation of caspase-3 can be seen as a switch between pro-inflammatory activation and apoptosis in microglia cells. cIAP2 hinders further processing of p19 to p17 in activated microglia, and entry to the nucleus. The cytoplasmic localization of caspase-3 promotes the pro-inflammatory activation and hinders apoptosis.



**Figure 7.** cIAP2 regulation of caspase-3 processing controls the switch between pro-inflammatory and cell death pathways.

## 3.2 PAPER II

### Deletion of caspase-8 in mouse myeloid cells blocks microglia pro-inflammatory activation and confers protection in MPTP neurodegeneration model.

The finding of caspases controlling microglia activation<sup>119</sup> encouraged us to provide the genetic evidence in a caspase-8 knockout mice model. Since sustained microglial pro-inflammatory activation is associated with the pathogenesis in neurodegenerative disorders, we decided to examine our genetic model in the context of neurodegeneration using a mouse model for Parkinson's disease (PD). The 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model has a significant inflammatory response and degeneration of dopaminergic neurons. In addition we used an acute LPS model, which also includes a strong inflammatory response and neurotoxicity in the substantia nigra.

The conditional knockout model of caspase-8 in myeloid cells was created by crossing mice bearing a floxed allele of CASP8 and a transgenic line expressing Cre under the control of the



Lysozyme 2 gene ( $\text{Cre}^{\text{LysM}}\text{Casp8}^{\text{fl/fl}}$ ). After intranigral injections of LPS or administration of MPTP, a significant decrease of cleaved caspase-8 is observed in Iba1 positive cells in the  $\text{Cre}^{\text{LysM}}\text{Casp8}^{\text{fl/fl}}$  mice as compared to the  $\text{Casp8}^{\text{fl/fl}}$  mice used as control, confirming the depletion of caspase-8 in the myeloid brain cells. Isolation of microglia cells from LPS injected and intact mice showed a low level of caspase-8 gene deletion in resident microglia, but a very high rate of caspase-8 gene deletion in the activated cells from the LPS mice.

Immunohistochemical analysis after intranigral injections of LPS showed equal numbers of Iba1-positive cells and marked changes in microglia morphology, indicating activation, in both the  $\text{Casp8}^{\text{fl/fl}}$  and  $\text{Cre}^{\text{LysM}}\text{Casp8}^{\text{fl/fl}}$  mice. Interestingly, there is also a clear downregulation of the pro-inflammatory marker CD16/32 detected in the caspase-8 deficient mice, indicating caspase-8 deletion hinders microglia activation in the LPS model. Additionally, the cleaved caspase-8 found in the  $\text{Casp8}^{\text{fl/fl}}$  mice was mostly restricted to CD16/32 labeled microglia. qPCR analysis of inflammatory mediators confirmed the upregulation of pro-inflammatory markers in the  $\text{Casp8}^{\text{fl/fl}}$  mice, but revealed significant prevention of the same markers in the  $\text{Cre}^{\text{LysM}}\text{Casp8}^{\text{fl/fl}}$  mice.

The results were then confirmed in the MPTP model of PD. We could detect similar numbers of microglia in the mesencephalon and striatum, but a significantly higher density of pro-inflammatory activated microglia in the  $\text{Casp8}^{\text{fl/fl}}$  mice as compared to the  $\text{Cre}^{\text{LysM}}\text{Casp8}^{\text{fl/fl}}$  mice. The caspase-8 activation in microglia was also detected mainly in the CD16/32 and Iba1 positive cells in the  $\text{Casp8}^{\text{fl/fl}}$  mice, but rarely in the  $\text{Cre}^{\text{LysM}}\text{Casp8}^{\text{fl/fl}}$  mice.

A quantification of the dopaminergic nigral neurons revealed a large reduction in Tyrosine hydroxylase (TH) positive neurons upon MPTP administration in the  $\text{Casp8}^{\text{fl/fl}}$  mice, but a smaller, close to significant, decrease in the  $\text{Cre}^{\text{LysM}}\text{Casp8}^{\text{fl/fl}}$  mice. However, analysis of the striatal dopaminergic nerve terminal density showed significant higher loss in the  $\text{Casp8}^{\text{fl/fl}}$  mice than the  $\text{Cre}^{\text{LysM}}\text{Casp8}^{\text{fl/fl}}$  mice in comparisons to the controls.

We could hereby conclude that caspase-8 gene deletion ( $\text{Cre}^{\text{LysM}}\text{Casp8}^{\text{fl/fl}}$ ) in myeloid cells demonstrated a high efficiency in activated but not in resident microglia. The caspase-8 deletion led to reduced pro-inflammatory activation of microglia upon LPS or MPTP treatment and was accompanied by a protective effect on the nigro-striatal dopaminergic system in the MPTP model.

### 3.3 PAPER III

#### **Temporal investigation of caspase-8 activation in myeloid cells in human stroke subjects.**

Based upon the earlier finding of caspase-8 and -3/7 regulating microglia activation<sup>119</sup>, we decided to investigate this caspase activation in microglia cells in stroke. Here we had the

opportunity to study their activation pattern in post mortem tissue from patients who had suffered an ischemic stroke.

Ischemic stroke is caused by a blockage or reduction in cerebral blood flow and leads to tissue damage and recruitment and activation of immune cells, including resident microglia and infiltrating macrophages. The ischemic core suffers the instant damage, which gradually spread to the surrounding penumbra unless the blood flow is rapidly restored and the inflammation is resolved<sup>120</sup>.

We started with immunohistochemical and immunofluorescent stainings of tissue from human stroke subjects. Antibodies detecting cleaved caspase-8 or -3 were used together with CD68, which detects activated microglia/macrophages (MMs). We could detect large numbers of CD68-positive MMs in the penumbra and ischemic core, and confirm the presence of active caspase-8 and -3 in the MMs.

Next, we wanted to understand the temporal and spatial expression of caspases after stroke. We investigated this aspect in a permanent middle cerebral artery occlusion (pMCAO) mouse model, where brain tissue was analyzed 6, 24 and 48 hours post occlusion. Already after 6 hours, morphological changes could be detected in Iba1-positive MMs in the penumbra. Morphological changes associated with MM activation were increasing at the 24 and 48 hours' time points. Here we choose to focus on the apical caspase-8. It could be detected at very low levels in Iba1-positive cells already at 6 hours, and showed an increased expression at the later time points. Interestingly, increasing levels of active caspase-8 correlated with the morphologically changes associated with activated MMs. Increased caspase levels and activation both became more prominent in the penumbra the closer the cells were to the ischemic core.

Finally, we were able to stain tissue from stroke patients who had suffered from two stroke events, one recent and one older. We discovered that expression of active caspase-8 and -3 could only be detected in the CD68 positive cells of the recent stroke area, but not in the older. Analysis of a panel of stroke patients with different ages of the stroke areas (n=9) and controls (n=5) suggested a correlation between presence of active caspases in CD68-positive cells and the age of the stroke area. The caspase expression decreased over time, along with decreasing numbers of CD68-positive cells.

The distinct pattern of active caspase-8 observed around the infarct area suggests that immunohistochemical stainings of cleaved caspase-8 could be used as an additional diagnostic marker for ischemic stroke.

In summary, our data illustrates a temporal and spatial activation of caspase-8 and -3 in microglia/macrophages after ischemic stroke. The caspase expression correlates with the time after stroke in analysis of the patient tissue and with the proximity of the infarct core, as shown in the pMCAO animal stroke model.

### 3.4 PAPER IV

#### Association with Alzheimer's disease and functional analysis of rare CASP8 variants.

In this paper we wanted to screen for mutations in genes involved in amyloid precursor protein (APP) metabolism, including caspase-8 and -3, in patients with late-onset AD to identify novel genes associated with the etiology of AD.

Dominant mutations in genes encoding APP and presenilin 1 and 2 are common causes of familial early-onset AD, and has recently been recognized as rare variants in late-onset AD as well<sup>63, 72, 121</sup>. The dysregulation of APP processing and accumulation of amyloid-beta (A $\beta$ ) are described as central events in the development of AD according to the amyloid cascade hypothesis<sup>63, 64</sup>. We were therefore interested in screening for other genes involved in APP metabolism, and included nine candidates in our study. We performed a rare variant association study using targeted sequencing in 1886 AD cases and 1700 controls. We also included APP, PSEN1, PSEN2 and TREM2 to identify known casual genes in our cohort to evaluate our study.

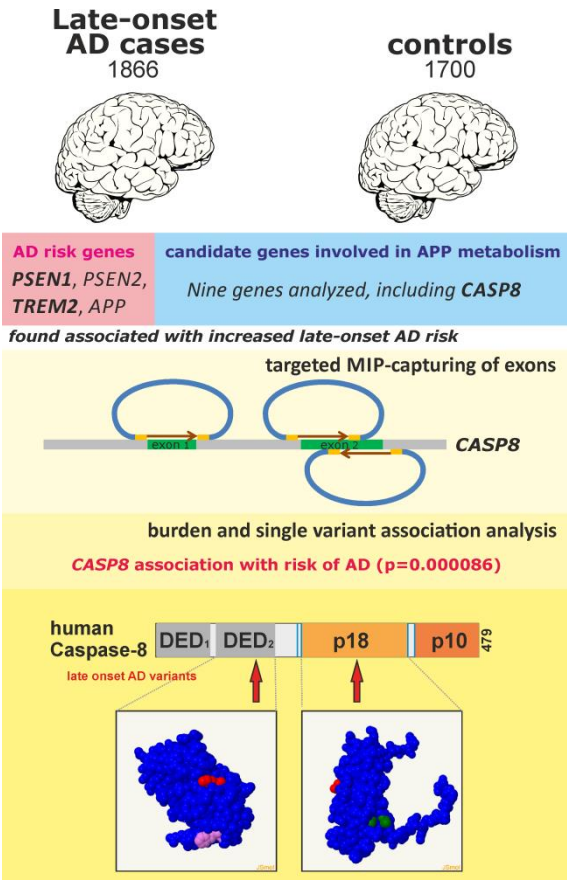
The variant-burden test reached nominally significant association for PSEN1, TREM2, APH1B and CASP8. PSEN1 showed strong association with 23/1883 rare variants in cases and 6/1700 in controls (burden test  $p=0.0027$ ). For TREM2, rare variants were observed in 114 cases and 52 in controls (burden test  $p=1.2 \times 10^{-5}$ ). For the candidate genes involved in APP metabolism, the APH1B rare variants was found in 17 cases and 6 controls ( $p=0.031$ ), but was not found to be significant when corrected for multiple testing. CASP8 however showed a significant association with AD in burden analysis after corrections for multiple tests. We observed rare variants in 26 cases and 4 controls ( $p=8.6 \times 10^{-5}$ , OR=5.93), which were all confirmed with capillary sequencing.

For CASP8 we found two protein coding variants that were overrepresented in AD cases, here called CASP8<sub>mut1</sub> and CASP8<sub>mut2</sub>. Structural analysis of the two variants shows that the substitutions affect exposed amino acid residues in death domain 2 within the prodomain and the large catalytic subunit of caspase-8 respectively.

We were then interested to see if the substitutions had an effect on caspase-8 function. Plasmids encoding for the two variants were transfected into caspase-8 defective neuroblastoma cells and evaluated in functional studies. The CASP8<sub>mut1</sub> variant showed higher accumulations of active caspase-8 in aggregate structures in immunofluorescence analysis and higher levels of caspase-8 and its proteolytic fragments with immunoblotting, as compared to CASP8 wildtype. For the CASP8<sub>mut2</sub> variant reduced levels of caspase-8 and processed fragments were shown. In addition, the CASP8<sub>mut2</sub> variant showed a significant reduction in caspase-8 activity (LETDase activity), while CASP8<sub>mut1</sub> did not appear to affect its enzymatic activity. However reduced activation (DEVDase activity) of caspase-3, a substrate and downstream effector of caspase-8, was observed for both CASP8 variants. Moreover, both CASP8 variants are able to process APP and generate the caspase cleaved

APPΔC31 fragment, as judged by immunoblot analysis. However, if they are as efficient as CASP8 wildtype remains unclear.

To summarize: In this study we used targeted sequencing and variant-burden test and thus identified a strong association of rare missense variants in the CASP8 gene with AD. Functional studies of two CASP8 variants showed altered expression of the caspase-8 protein and effect on caspase-8 and caspase-3 activity. The reduced enzymatic activity may have effect on several cellular processes that are regulated through caspase activity and contribute to brain homeostasis, which justifies further studies of caspase-8 role in the etiology of late-onset AD.



**Figure 8.** Screening of a large cohort of AD patients and controls by targeted sequencing identified a strong association of rare missense variants in the CASP8 gene with AD. Mutations in CASP8 gene are indicated by red arrows.

## 4 DISCUSSION

The studies in this thesis have focused on the roles of caspase-8 and -3 in microglia activation and in disease. Several projects were based on a previous finding in the group of novel roles for caspases in microglia activation. Upon pro-inflammatory stimulus, activation of caspase-8 and -3 were shown to regulate microglia activation, in the absence of cell death. This discovery raised many new questions: These caspases are well known for their role in apoptosis, but here they are not killing the cells. What is preventing them from their apoptotic functions? Can we present genetic evidence of caspase involvement in microglia activation? Can we detect these caspases in activated microglia in disease?

In order to answer these questions we started by focusing on the apical caspase-8. In **Paper II** we wanted to provide genetic evidence for the caspase regulation of microglia activation. We created a conditional knock-out of caspase-8 in microglia cells using the Cre-Lox system in mice. Knock-out of caspase-8 reduced the microglia pro-inflammatory phenotype and showed a reduction in neuronal cell death in a Parkinson's disease model. It is interesting to speculate that targeting these caspases could be used as a strategy to reduce microglia pro-inflammatory activation and neurotoxicity in disease.

In **Paper I** we wanted to understand why the caspases are not killing the cells during microglia activation. Here we describe a mechanism for how cIAP2 can act as a molecular switch between pro-inflammatory- and cell death pathways in microglia by regulating the processing of caspase-3. In this paper we also use a SMAC mimetic, the BV6 compound, to reduce cIAP2 inhibition of caspase-3 processing. Through this mechanism, treatment of microglia with the BV6 compound resulted in reduced microglia activation. However, if reducing the number of pro-inflammatory activated microglia by induction of cell death is beneficial in disease remains to be investigated. Caspase inhibitors such as e.g. Q-VD-OPh might be a more direct approach for therapeutic purposes. Still, the effect on other signaling pathways and other cell types in the brain has to be taken into account as well.

Another important question to be asked is in which phase of the disease it is beneficial to target caspases in microglia activation? In **Paper III** we investigated the temporal and spatial expression of caspase-8 and caspase-3 in activated microglia after stroke. We examined the caspase expression in a panel of stroke patients, and concluded that detection of caspases correlated with the age of the ischemic area. If considering using caspases as a way to target microglia activation, one has to consider this time-window for interventions.

In **Paper IV** we identified two rare variants of CASP8 associated with late-onset Alzheimer's disease. We also showed *in vitro* that these mutations could lead to a reduction in enzymatic activity in a neuroblastoma cell line. Although a moderate change, reduced activity over a lifetime (as in late-onset AD) could have consequences that are accumulating over time. Considering the roles of caspase-8 and its downstream effector caspase-3 in several cellular processes, the altered enzymatic activity can affect the different cell types in many ways. Here we have studied the effect of overexpression of the mutated caspases after 24 hours. In

the future it would be very interesting to use knock-in CASP8 mutations in cell lines or in an *in vivo* model and study their long-term effect. It would also be interesting to study the CASP8 mutations in microglia cells to investigate their effect on microglia activation.

In summary; in this thesis we studied the role of caspase-8 and -3 in microglia activation and disease. We provided genetic evidence for caspase-8 regulation of microglia pro-inflammatory activation (**Paper II**) and described a mechanism for how caspase-3 is prevented of killing the cells during activation (**Paper I**). We also described the expression pattern of those caspases in microglia cells after stroke (**Paper III**), and provided evidence of rare mutations in caspase-8 associated with late-onset Alzheimer's disease (**Paper IV**).

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